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Research Article

SUGARS MEDIATED GERMINATION IN SPORES OF *Bacillus megaterium*

TEHRI NIMISHA*, KUMAR NARESH, YADAV AVINASH, RAGHU H.V. AND SINGH NAMITA ASHISH

Microbial Biosensors and Food Safety Laboratory, Dairy Microbiology Division, ICAR-National Dairy Research Institute, Karnal 132001, Haryana, India
*Corresponding Author: Email-nimmi.tehri15@gmail.com

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Abstract- A total of eleven sugars were evaluated for their potential to endow germination in spores of *Bacillus megaterium* MTCC 2949. Germination of spores in response to different sugars was studied by two well-known germination assays namely reduction in O.D. (600nm) and refractility examination. As a result, reduction in O.D. (600 nm) varied from ~3.90 to 42.01% depending upon type and concentration of different sugars. Spores were found germinated well with a reduction in absorbance of 30% or more with cellobiose, dextrose, 2-deoxy-D-glucose, glucosamine, maltose, methyl-α-D- glucoside, sorbose, starch and xylose. On the other hand, germination was found at a poor or negligible rate (*i.e.*, < 6% reduction of absorbance) with D-galactose and rhamnose. The finding of this assay was further validated by refractility study using phase contrast microscopy. The outcome of presented work reveals that all sugars are not necessarily capable of inducing germination in bacterial spores and hence induction of germination is linked with the requirement of specific sugars.
Key words- *Spore, Germinant, Germination, Sugars, Refractility*

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Introduction

Many species of Gram positive and Gram negative bacteria such as Bacillus, Clostridium, Sporomusa and Coxiella are able to form dormant and resistant spores under unfavourable environmental conditions. Spores can germinate in response to favourable environment conditions and presence of germinants that trigger germination followed by outgrowth to generate growing vegetative cells [1-3]. Germinants can be of nutrient and non-nutrient types. The germination of bacterial spores is usually triggered by nutrient germinants. These are generally low molecular weight compounds and are species- and strain specific (4). Germination in spores can be triggered by a wide variety of compounds of quite diverse nature, including glucose, L-alanine, or KNO_3 [5-7]. However, the specificity of the spores for germinants is due to the proteins, termed as 'germinant receptors' (GR) [8]. The presented work reports the observations with eleven sugars that have been examined for their ability to support germination in *Bacillus megaterium* spores.

Materials and Methods

Bacterial culture: *Bacillus megaterium* strain MTCC 2949 was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The culture was revived by inoculation in nutrient broth and incubated overnight at 37ºC. Following incubation loopful of the culture was streaked on nutrient agar and incubated at 37°C for 12-16 h for further use in experimentation.

Production of bacterial spores: Spores in *Bacillus megaterium* were produced by using nutrient starvation principle based method [9, 10]. Loopful of fresh culture of *B. megaterium* from nutrient agar medium (Himedia, India) was inoculated in trypton glucose yeast extract broth, propagation medium and incubated at 37°C for 24 h. One mL of developed inoculum was transferred to 100 mL propagation medium and further incubated at 37°C for 48 hrs. Culture grown was transferred to 100 mL of sporulation medium with less nutrient concentration and incubated at 37°C for 42 h. Following incubation, washing of spores was performed with potassium phosphate buffer (10mM, pH=6.8) and final suspension of spores with an O.D. (600 nm.) of 0.330±0.015 (107 spores/ mL) was prepared in same buffer and stored under refrigeration at 2–8˚C. Each spore batch was also evaluated for total viable count and spore count (11). Formation of spores was further confirmed by refractility examination using phase contrast microscopy (Olympus BX 51 photomicroscope equipped with UPLAPO PH series of objectives, phase-contrast condenser U-PCD2 and digital camera DP70).

Spores activation: Spore suspension (10^7 spores/ mL) was taken in a thin-walled glass tube and incubated at 80°C for 10 min. in AccuBLOCK digital dry bath (Labnet International Inc., U.S.A.). Heat treatment step was followed by rapid cooling of spores using cold water.

Preparation of sugars: Sugars, as given in [Table-1], of analytical grade (typically > 98% pure) were procured from Sigma Aldrich (India). Six different concentrations *i.e.*, 50, 100, 150, 200, 250 and 300 (mM) of each sugar were prepared in sterile 10 mM potassium phosphate buffer (pH = 6.8). Further each sugar solution was sterilized by filtration using 0.22 µm membrane filters.

Spore germination studies: Germination in spores of *B. megaterium* in response to eleven sugars, was assessed by monitoring O.D. (600 nm) and refractility examination [10]. Each of these assays was performed with spores at an O.D. (600 nm) of 0.330±0.015.

Table-1 *List of Sugars*

Sugar	Molecular Formula
Cellobiose	$C_{12}H_{22}O_{11}$
Dextrose	$C_6H_{12}O_6$
2-deoxy-D-Glucose	$C_6H_{12}O_5$
Glucosamine	$C_6H_{13}NO_5$
Galactose	$C_6H_{12}O_6$
Methyl-α-D- glucosidase	$C_7H_{14}O_6$
Maltose	$C_{12}H_{22}O_{11}$
Rhamnose	$C_6H_{12}O_5$
Sorbose	$C_6H_{12}O_6$
Starch	$(C_6H_{12}O_5)n$
Xylose	$C_5H_{10}O_5$

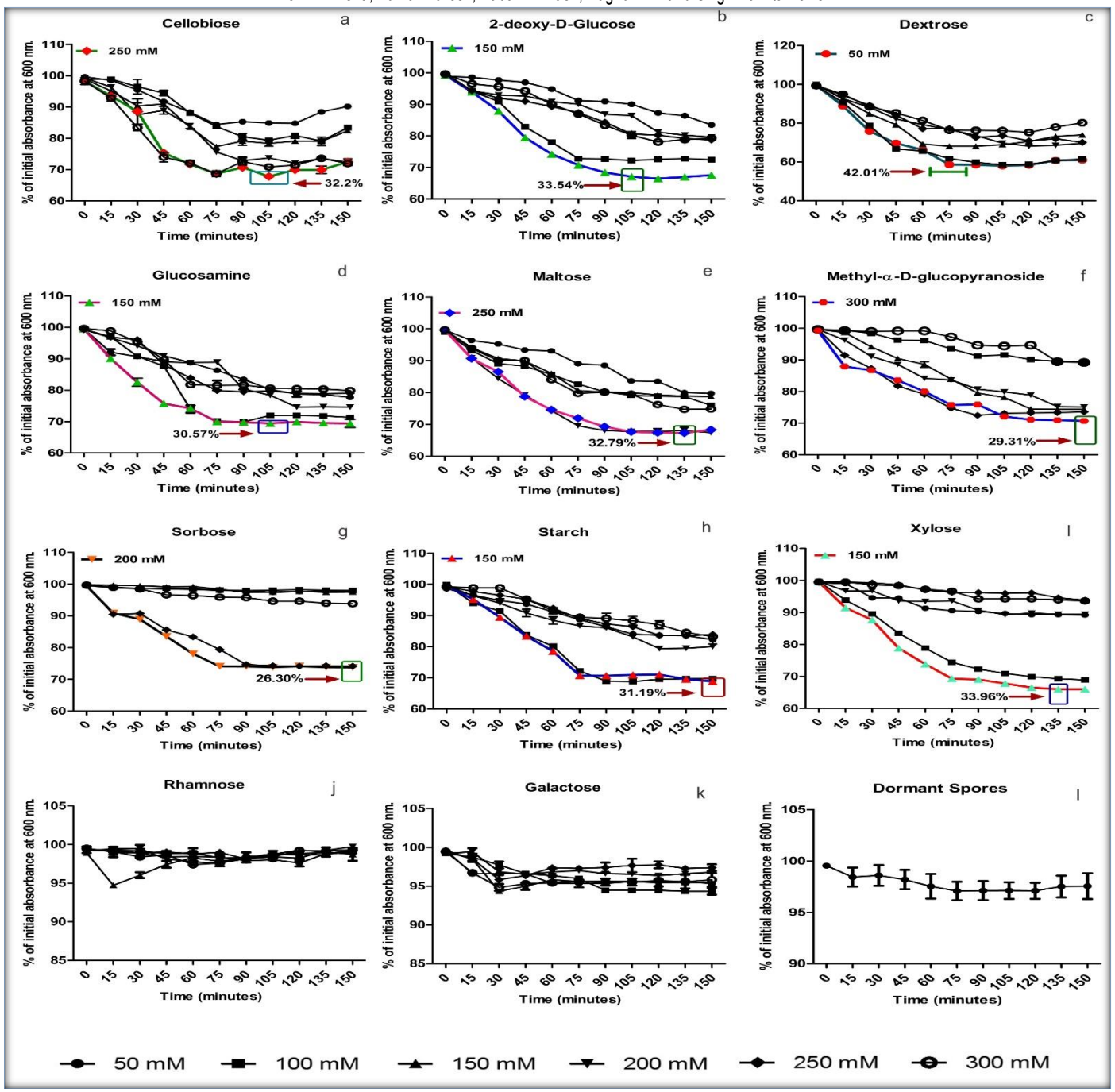

Fig-1 (a-l). Reduction in O.D. of *B. megaterium* MTCC 2949 spores in response to different sugars: a-cellobiose, b-2-deoxy-D-glucose, c- dextrose, d- glucosamine, e- maltose, f- methyl α-D-glucoside, g-sorbose, h-starch, i-xylose, j-rhamnose, k-galactose, l-dormant spores (Error bars in graph represents standard error, n=3).

In O.D. based approach, germination in response to different sugars was assayed by adding 75 µL of heat activated spores to 75 µL of sugar (50-300 mM) in microtitre plate (M/s Grenier, Germany). The control of dormant spores and potassium phosphate buffer were also included. O.D. at 600 nm was determined after every 15 min. for a period of 150 min. The germination in spores was measured in terms of total decrease in O.D. at 600 nm. Refractility of spores was studied using phase contrast microscope. For microscopic examination, 100µL each of spores and sugar (at concentration of sugar that showed maximum reduction in O.D. 600 nm) were taken in microcentrifuge tubes. The controls of spores and buffer were also included. Incubation was carried out at 37˚C for 3.0 h. After incubation, reaction mixture containing spores and sugar was spread on a clean glass slide to make thin smear. Smears were air dried and heat fixed to minimize the loss of the spores (dormant or germinated)/cells. Refractility was examined to study germination in spores of *B. megaterium* in response to different sugars.

Results and Discussion

Screening of sugars as germinants for *Bacillus megaterium* spores

A total of eleven sugars [Table-1], were screened for their potential to endow germination in spores of *B. megaterium* MTCC 2949. Germination in response to these sugars was studied using well known markers of spore germination *i.e.*, decrease in O.D. (600 nm.) and loss of refractility. The findings obtained on germination response of spores against different sugars are described below.

Reduction in O.D. (600 nm)

The maximum rate of reduction in the O.D. (600 nm) at a given germinant concentration, is a simple and reliable method for comparing rates of spores germination [12]. This principle was used to evaluate the efficiency of sugars for triggering germination in bacillus spores. As a result, nine out of eleven sugars, investigated in the current study, indicated the onset of germination at significant level in MTCC 2949 spores. Findings on O.D. (600 nm.) reduction of spores incubated with different concentrations of sugars (50-300 mM) at varying intervals of time for a period of 150 min. are shown in [Fig-1] (a-l). Reduction of O.D. (600 nm.) varied from ~3.90 to 42.01 % in a period of 150 min. depending upon type and concentration of sugar.

Spores found germinated well with a reduction in absorbance of 30% or more with cellobiose (250 mM), dextrose (50 mM), 2-deoxy-D-glucose (150 mM), glucosamine (150 mM), maltose (250 mM), methyl-α-D- glucoside (300 mM), sorbose (200 mM), starch (150 mM) and xylose (150 mM). This significant fall in absorbance reflects germination in terms of change in the light scattering property of the multiple individual spores in a suspension, associated with germination events such as the excretion of spore's depot of Ca^{2+}-DPA, followed by water influx, cortex degradation and core swelling [13-15]. On the other hand, spores found germinated poorly or to a negligible rate (*i.e.*, < 6% reduction of absorbance) with D-galactose, Rhamnose. These findings were similar to the O.D. (600 nm.) loss *i.e.*, < 5% shown by dormant spores incubated without sugar and thus proved their non-germinant characteristic. Similarly, germination in spores of *B. megaterium* QM B1551 in response to D-glucose, glucosamine, 2-deoxy-D-glucose, sorbose, L-alanine and L-proline while no germination in response to rhamnose and galactose was also reported in other work which supports the findings obtained in presented investigation [16, 17, 10].

Refractility examination

Refractility in spores supplemented with concentration of each sugar that showed maximum loss of O.D. at 600 nm was studied using phase contrast microscopy. As a result, spores incubated with optimized concentrations of cellobiose, dextrose, 2-deoxy-D-glucose, glucosamine, maltose, methyl-α-D-glucoside, sorbose, starch, and xylose induce the triggering of spore's germination. This was proved by the rapid loss of refractility, as viewed in the images shown in [Plate-1.0 (a-i)]. This change in spore's refractive index occur dramatically during germination due to both the release of the spore core's large DPA pool and some water uptake, as well as subsequent cortex hydrolysis that leads to core swelling and further water uptake [18,19].

In contrast, when spores incubated with D-galactose and Rhamnose did not show any sign of germination [Plate-1.0, j-k]. These spores were quite refractile in appearance similar to dormant spores as depicted in [Plate-1.0 (l)] and thus indicated lack of the spore germination process.

Based on these observations, nine sugars exhibited rapid loss of refractility in spores and hence showed germination response. These findings simulate other works that reported a rapid loss in characteristic refractility of spores during triggering of germination process resulting in their dark appearance when examined by the phase-contrast microscopy [20].

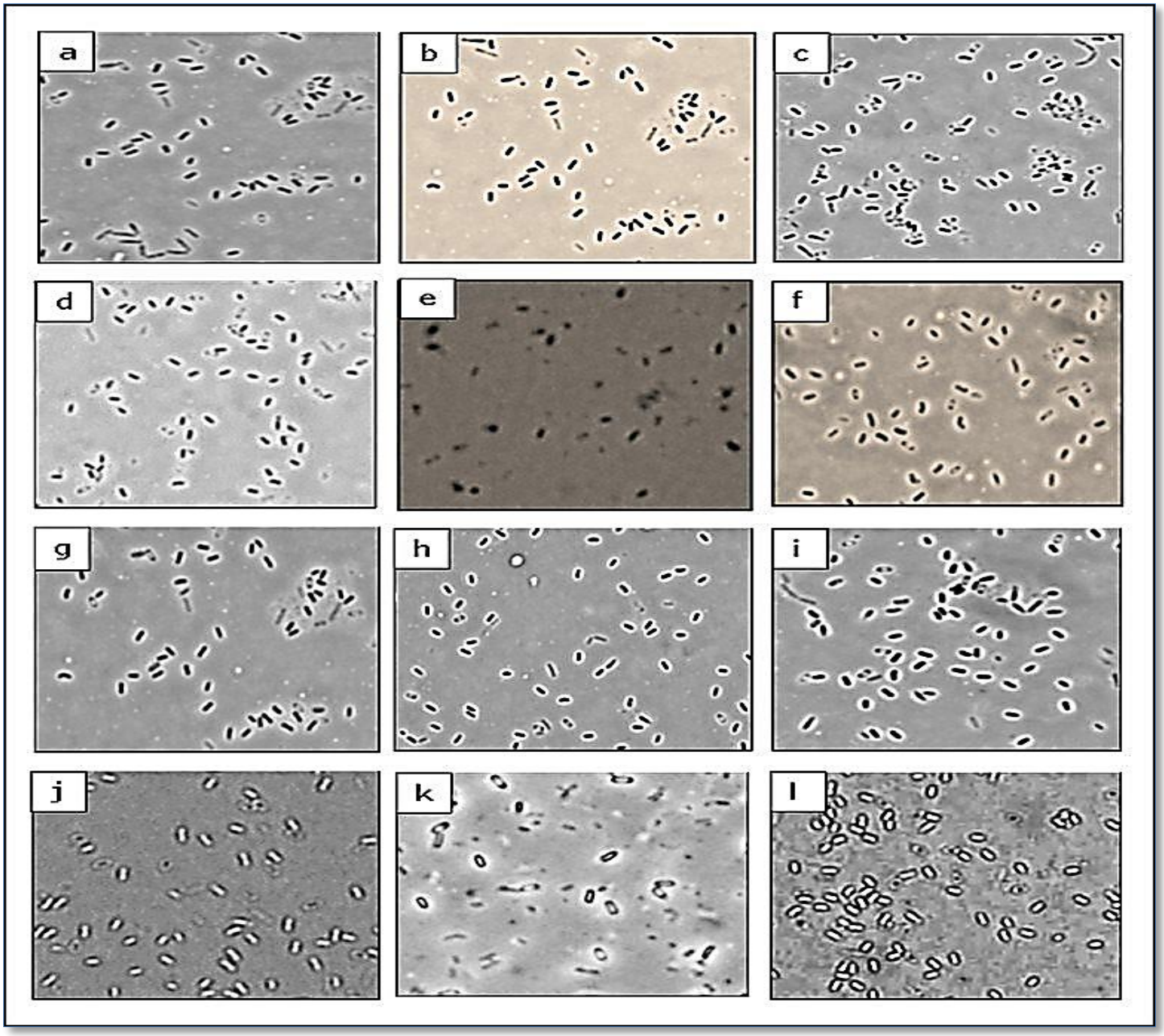

Plate-1 (a-l). Phase contrast microscopic observations of *B. megaterium* MTCC 2949 spores: a-cellobiose, b- dextrose, c-2-deoxy-D-glucose, d- glucosamine, e- maltose, f- methyl α-D-glucoside, g-sorbose, h-starch, i-xylose, j-rhamnose, k-galactose, l-dormant spores

The findings on spore's germination with different sugars by O.D. (600 nm.) and refractility based approach were found correlated to each other. [Table-2] summarizes the findings of these germination assays. Sugars which showed significant reduction in O.D. (600 nm.) equivalent to 30% or more and corresponding loss in refractility of spores were reported as potential germinant for spores of MTCC 2949 strain. Current work depicts the specific requirement of sugar to trigger germination in spores. This distinction in recognition of sugars by spores can be speculated to link with permeability of the outer spore layers, for different germinant to the inner membrane [21], germinant specificity [22, 23] and GR (nutrient germinant receptors) level [15]. Germination in response to multiple germinants is consistent with existing explained strategies [2] according to which multiple germinants can interact with a single receptor, and any one of the cognate germinants is sufficient to trigger germination. Most of the sugars reported for their germination potential for spores of *B. megaterium* in present investigation is consistent with the findings obtained on Bacillus spores germination of other workers also [24].

Table-2 *Summary on findings of germination assays for screening of germinants*

Sugars	Germination response		Nature Revealed
	% Reduction in O.D. (600 nm.)	Refractility under Phase contrast microscopy	
Cellobiose	32.20	Non-refractile, Phase dark	Germinant
Dextrose	42.01	Non-refractile, Phase dark	Germinant
2-deoxy-D-Glucose	33.54	Non-refractile, Phase dark	Germinant
Glucosamine	30.57	Non-refractile, Phase dark	Germinant
Galactose	5.66	Refractile, Phase bright	Non-Germinant
Maltose	32.70	Non-refractile, Phase dark	Germinant
Methyl-α-D-glucopyranoside	29.31	Non-refractile, Phase dark	Germinant
Rhamnose	5.66	Refractile, Phase bright	Non-Germinant
Sorbose	26.30	Non-refractile, Phase dark	Germinant
Starch	31.19	Non-refractile, Phase dark	Germinant
Xylose	33.96	Non-refractile, Phase dark	Germinant

Conclusion

Bacillus megaterium MTCC 2949 is a non-pathogenic spore former having great potential for its use in development of sensing technologies for targeting analytes like heavy metals, pesticides, adulterants etc. In current work, sugars with potential to act as germinant for spores of this strain have been studied that can find great applications of same in future. In addition, the outcome of presented work reveals that all sugars are not necessarily capable of inducing germination in bacterial spores and hence induction of germination is linked with the requirement of specific germinants. The conclusions drawn from current work can be helpful in understanding the role of germinants in germination onset, germination mechanism that can further contribute immensely in the field of development of spore eradication and spore based biosensing tools.

Application of research

Presented work on sugars mediated germination of bacterial spores can be helpful in understanding the mechanism of spore germination concept and its use for development of spore-based sensing to target different analytes (like pesticides, antibiotics, heavy metals etc.) of interest in future.

Research Category: Bacteriology, Bacterial spores.

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Abbreviations:
O.D.: Optical density
GR: Germinant Receptors
Ca^{2+}-DPA: Calcium Dipicolinic acid
MTCC: Microbial Type Culture Collection

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